Title Angiotensin-III is increased in Alzheimer's disease in association with amyloid-beta and tau pathology

Running title Elevated Ang-III in Alzheimer's disease

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Abstract

Hyperactivity of the renin-angiotensin system (RAS) is associated with the pathogenesis of Alzheimer's disease (AD) believed to be mediated by angiotensin-II (Ang-II) activation of the angiotensin type 1 receptor (AT1R). We previously showed that angiotensin-converting enzyme-1 (ACE-1) activity, the rate-limiting enzyme in the production of Ang-II, is increased in human post-mortem brain tissue in AD. Angiotensin-III (Ang-III) activates the AT1R and angiotensin type-2 receptor (AT2R) but its potential role in the pathophysiology of AD remains unexplored. We measured Ang-II and Ang-III levels by ELISA, and the levels and activities of aminopeptidase-A (AP-A) and aminopeptidase-N (AP-N) (responsible for the production and metabolism of Ang-III respectively) in human post-mortem brain tissue in the mid-frontal cortex (Brodmann area 9) in a cohort of AD (n=90) and age-matched non-demented controls (n=59), for which we had previous measurements of ACE-1 activity, Aβ level and tau pathology (also in the mid-frontal cortex). We found that both Ang-II and Ang-III levels were significantly higher in AD compared to age-matched controls and that Ang-III, rather than Ang-II, was strongly associated with Aβ load and tau load. Levels of AP-A were significantly reduced in AD but AP-A enzyme activity was unchanged whereas AP-N activity was reduced in AD but AP-N protein level was unchanged. Together, these data indicate that the APA/Ang-III/APN/Ang-IV/AT4R pathway is dysregulated and that elevated Ang-III could contribute to the pathogenesis of AD.

Keywords: Angiotensin-III; angiotensin-II; aminopeptidase-A; aminopeptidase-P; renin-angiotensin system; Alzheimer's disease

Introduction

It is now well established that there is a local-acting renin-angiotensin-system (RAS) within the brain that functions independently from the systemic RAS [1-4] and that hyperactivity of brain RAS is associated with the pathogenesis of Alzheimer's disease (AD) [5]. Most of the deleterious effects of the brain RAS in AD are believed to be associated with elevated Ang-II levels and its subsequent over-activation of angiotensin receptor type-1 (AT1R), commonly referred to as the classical axis of RAS (reviewed in [5]). Infusion of Ang-II increased both amyloid-beta (Aβ) (via increased amyloidogenic processing of APP) and tau pathology, and reduced cognitive performance in ageing Sprague Dawley rats [6, 7]. We have previously reported that angiotensin-converting enzyme-1 (ACE-1), the rate-limiting enzyme in the production of Ang-II, is increased in AD in human brain tissue [8, 9]. Epidemiological studies suggest that commonly prescribed anti-hypertensives that target brain RAS and thus reduce the activity of classical RAS (ACE-1 inhibitors and AT1R blockers) protect against cognitive decline and that re-purposing these drugs may have therapeutic potential in AD [10, 11].

Angiotensin-III (Ang-III) is a heptapeptide produced following aminopeptidase-A (AP-A) (EC 3.4.11.7) mediated cleavage of the Asp residue at the N-terminal of Ang-II [12-15]. Ang-III is a potent activator of AT1R and AT2R [16, 17]. Evidence from experimental animal models suggest that Ang-III shares many of the biological effects that are attributed to Ang-II and that Ang-III-mediated activation of AT1R is implicated in a host of responses including increased blood pressure [18-21], dysregulated drinking and salt intake [20], increased pituitary hormone release [22, 23] and increased vasopressin release [24]. Orally administered AP-A inhibitors, resulting in Ang-III reduction, are cardioprotective [25-28] suggesting that Ang-III, rather than Ang-II, may be the central effector protein in RAS (reviewed in [3]). Ang-III is metabolised by aminopeptidase-N (AP-N) (EC 3.4.11.2) to generate Ang IV [29], a peptide that via inhibition of insulin-regulated aminopeptidase (IRAP), is heavily implicated in learning and memory consolidation [30, 31]. This APA/Ang-III/APN/Ang-

IV/AT4R pathway remains poorly characterised within the brain and its potential role in AD is poorly understood. Recent studies indicate that serum and plasma AP-A and AP-N activities are reduced in early AD [32, 33].

In the present study, we have measured the levels of Ang-II and Ang-III, and the levels and activities of AP-A and AP-N (responsible for the metabolism of Ang-II and Ang-III respectively) in relation to Aβ and Tau pathology in AD.

Materials and Methods

Case selection

Brain tissue was obtained from the South West Dementia Brain Bank, University of Bristol, with local research ethics committee approval. All brains had been subjected to detailed neuropathological assessment according to the NIA-AA guidelines [34] and AD pathology was a sufficient explanation for the dementia in these cases. We investigated 90 cases of AD and 59 age-matched controls whose brains were from people who had no history of dementia and had undergone extensive neuropathological assessment and had few or absent neuritic plaques, a Braak tangle stage of III or less, and no other neuropathological abnormalities. All cases had previous measurements of ACE-1 activity [8, 35], total soluble (NP-40-extracted) and insoluble (Guanidine Hydrochloride (GuHcl)-extracted) Aβ levels [36], and tau load (area fraction of cerebral cortex immunopositive for ptau) [37, 38] within the mid-frontal cortex (Brodmann area 9). The cohorts were matched closely for age-at-death and post-mortem delay. The demographic data are presented in Table 1 and the MRC UK Brain Bank Network database identifiers are shown in Supplementary Table 1.

Brain tissue

The right cerebral cortex had been fixed in 10% formalin for a minimum of three weeks before the tissue was processed and paraffin blocks were taken for pathological assessment. The left cerebral hemisphere had been sliced and frozen at -80°C until used for biochemical assessment. For each case we dissected 200mg brain tissue from the mid-frontal cortex (Brodmann area 9) that was homogenised in a Precellys homogeniser (Stretton Scientific, Derbyshire, UK) as previously described [8, 9]. Brain homogenates were prepared in either 1% SDS lysis buffer (100uM NaCl, 10mM Tris pH 6, 1µM phenylmethylsulfonyl fluoride (PMSF), 1µg/ml aprotinin (Sigma Aldrich) and 1% SDS in distilled water) or 0.5% triton-X100 lysis buffer (20mM Tris pH 7.4, 10% sucrose, 1µM PMSF, 1µg/ml aprotinin (Sigma Aldrich) and 0.5% triton X-100 in distilled water). The samples were centrifuged at 13,000rpm and the clarified supernatants were aliquoted and stored at −80°C until required. Total protein was measured using the Total Protein kit (Sigma Aldrich, Dorset, UK) following manufacturer's guidelines. Most brain tissue was obtained within 72 hours of death.

Angiotensin-II sandwich ELISA

Ang-II level was measured in brain tissue homogenates (in 1% SDS lysis buffer) using a commercially available sandwich ELISA (Abcam, UK) following manufacturer's guidelines. In brief, recombinant human Ang-II or brain tissue supernatants (50 µl + 50µl PBS) were added in duplicate to wells that had been pre-coated with an anti-human Ang-II specific antibody and incubated for 2 hours at room temperature. After a wash step, the plate was incubated for 2 hours with a biotinylated anti-Ang-II detection antibody at room temperature. The plate was again washed followed by 30 minutes incubation with streptavidin:horse radish peroxidase (strep:HRP). After a final wash, TMB substrate was added for 20 minutes in the dark and the absorbance was read at 450nm using a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, Buckinghamshire, UK). The concentration of Ang-II was interpolated from a serial dilution of recombinant Ang-II (1,000-62.5pg/ml) and measured in duplicate for each case. Ang-II level is expressed as pg/mg total protein for each case.

Angiotensin-III direct ELISA

Ang-III level was measured in human brain tissue homogenates (in 1% SDS lysis buffer) using a direct ELISA. Recombinant human Ang-III (Sigma Aldrich, Dorset, UK) or human brain tissue homogenates (diluted 1 in 10 In PBS) were incubated for 2 hours in a clear high-binding capacity NUNC maxisorp plate (ThermoFisher Scientific, Waltham, MA, USA) at 26°C with shaking. The wells were washed five-times in PBS with 0.05% tween-20 and blocked for 1 hour in 1 % PBS: BSA (Sigma Aldrich, Dorset, UK). After another five washes, the wells were incubated with biotinylated anti-human Ang-III (diluted to 2ug/ml in PBS) (Cloud-Clone, Wuhan, China) for 2 hours at 26°C with shaking, followed by a further wash step. The plate was incubated with strep:HRP (1:200) in PBS:0.01% Tween-20 at room temperature for 20 minutes in the dark. TMB substrate (R&D systems) was added after a further wash and left to develop in the dark for 20 minutes. Absorbance at 450nm was read following the addition of 2N sulphuric acid ('stop' solution) using a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, BUCKS, UK). Ang-III concentrations were interpolated from a standard curve generated by serially diluting recombinant human Ang-III (20,000-312.5pg/ml) and were measured in duplicate. Ang-III level is expressed as ng/mg total protein. The assay showed minimal cross reactivity with a number of closely-related peptides including Ang I, Ang-II and Ang 1-7.

Aminopeptidase-A sandwich ELISA

AP-A level was measured in brain tissue homogenates (in 1% SDS lysis buffer) using a commercially available competitive sandwich ELISA (FIRELISA; Human GluAP Cat. No. ELISAHu004197). In brief, brain tissue homogenates (diluted 1 in 25 in PBS) or recombinant human AP-A was added together with biotinylated antigen for 1 hour at 37°C. After 5 washes with the proprietary wash buffer, streptavidin-HRP was added to each well for 1 hour at 37°C. After a further five washes, TMB chromogen was added to each well and incubated for 20 minutes in the dark. Absorbance at 450nm was read after the reaction was stopped using 2N sulphuric acid using a FLUOstar OPTIMA plate

reader (BMG labtech, Aylesbury, BUCKS, UK). AP-A levels were measured in duplicate for each case and were interpolated from a serial dilution of recombinant human AP-A (32-2ng/ml). AP-A level is expressed as ng/mg total protein.

Aminopeptidase-A enzyme activity assay

AP-A enzyme activity was measured in brain tissue homogenates (in 0.5% triton X-100 lysis buffer) using a fluorogenic peptide substrate, H-Glu-AMC-OH (AMC = 7-amido-4-methylcoumarin) (200uM) (Bachem, Bubendorf, Switzerland). The assay was performed in Nunc F16 black maxisorp 96-well plates (ThermoFisher Scientific, Waltham, MA, USA). A series of preliminary experiments were performed to test a range of substrate concentrations (400-25µM) using a serial dilution of recombinant human AP-A (400 – 3.2 ng/ml) over an extended reaction time i.e. fluorescence was read every 5 minutes up to 30 minutes and then again at 1 and 2 hours. H-Glu-AMC-OH at 200µM produced the highest fluorescent signal that became saturated at one hour i.e. total cleavage of substrate was observed. All subsequent experiments were performed using H-Glu-AMC-OH at 200µM and the reaction was allowed to proceed for 1 hour at 37°C. Brain tissue homogenates (triton X-100) (100ug total protein), or recombinant AP-A (serially diluted to produce a standard curve (400 – 3.2 ng/ml)) were diluted in assay buffer (25mM Tris pH 8.0, 50mM CaCl2, 200uM NaCl) and incubated in the presence of amastatin-HCl a general aminopeptidase inhibitor [39] (10uM) (Enzo Life Sciences, Exeter, UK), or assay buffer alone, for 10 mins at 37°C prior to the addition of H-Glu-AMC-OH (200uM diluted in assay buffer). The reaction was incubated at 37°C for 1 hour in the dark and fluorescence was read at an excitation/emission of 390/450nm using a using a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, BUCKS, UK). Enzyme activities are expressed as mMol of H-Glu-AMC-OH hydrolyzed per hour per mg of total protein (using the 7-amido-4-methylcoumarin (AMC) (Sigma Aldrich, Dorset, UK) as a reference).

Aminopeptidase-N sandwich ELISA

AP-N level was measured in brain tissue homogenates (in 1% SDS lysis buffer) using a commercially available ELISA kit (AP-N duoset, Cat no. DY3815) (R&D systems, Cambridge, UK). High-binding capacity NUNC Maxisorp 96-well plates (ThermoFisher Scientific, Waltham, MA, USA) were coated with a human anti-AP-N capture antibody diluted in PBS (0.8ug/ml) overnight at room temperature. The wells were washed five time in PBS/0.05% tween-20 and blocked with 1% PBS: BSA for 1 hour at room temperature. The wells were washed again and recombinant human AP-N (20-0.3125 ng/ml), or brain tissue (diluted 1 in 20 in PBS), was incubated for 2 hours at room temperature. After a further five washes, the wells were incubated with streptavidin:HRP (diluted 1:200) (R&D systems) for 20 mins in the dark. The wells were washed again and TMB substrate (100ul) (R&D systems) was added and left in the dark for 20 minutes. Upon addition of 2N sulphuric acid (50ul 'stop' solution) absorbance was read at 450nm in a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, BUCKS, UK). The concentration of AP-N was interpolated from a serial dilution of recombinant human AP-N and AP-N levels were measured in duplicate for each case and expressed as ng/mg total protein.

Aminopeptidase-N enzyme activity

AP-N enzyme activity was measured in brain tissue homogenates (in 0.5% triton X-100 lysis buffer) using an immunocapture-based activity assay using the fluorogenic peptide substrate, H-ALA-AMC (AMC = 7-amido-4-methylcoumarin) (200uM) (Bachem, Bubendorf, Switzerland). A black high binding-capacity Nunc maxisorp 96-well plate (ThermoFisher Scientific, Waltham, MA, USA) was coated overnight at room temperature with capture antibody from the AP-N duoset ELISA kit (0.8ug/ml)(R&D systems, Abingdon, Oxford, UK). After five washes in 0.05% PBS:Tween-20 each well was blocked in 1% PBS:BSA for 1 hour at room temperature. After another wash step, a serial dilution of recombinant human AP-N (200 – 1.56 ng/ml) (R & D systems, UK) or brain tissue homogenates (125ug total protein) were incubated for 2 hours at room temperature. After another 5 washes, wells were either incubated with betstatin-HCl (20uM) (Sigma Aldrich, Dorset, UK) diluted in assay buffer (50mM Tris, pH 7.0), or assay buffer alone, at 37°C for 10mins prior to the addition of

H-ALA-AMC (200uM). A series of preliminary experiments were performed to test a range of H-ALA-AMC concentrations (400-25µM) using a serial dilution of recombinant human AP-N (200 – 12.5ng/ml) over an extended reaction time i.e. fluorescence was read every 5 minutes up to 30 minutes and then again at 1 and 2 hours. H-ALA-AMC at 200uM produced the highest fluorescent signal that became saturated at one hour i.e. total cleavage of substrate was observed. All subsequent experiments were performed using H-ALA-AMC (200uM), the reaction was allowed to proceed for 1 hour at 37°C, and fluorescence was read at excitation/emission at 390/450nm using a FLUOstar OPTIMA plate reader (BMG labtech, Aylesbury, Buckinghamshire, UK). AP-N enzyme activity is expressed as mMol of H-ALA-AMC hydrolyzed per hour per mg of total protein (using the 7-amido-4-methylcoumarin (AMC) as a reference).

AP-A and AP-N immunohistochemistry

Paraffin sections (7µm) were dewaxed in Clearene, dehydrated in 100% ethanol and immersed in 3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. The tissue was washed in running tap water and sections were boiled in 9mM tri-sodium citrate, pH 6, for 5 minutes, left to stand for 5 minutes, boiled again for 5 minutes and finally left to stand for 15 minutes at room temperature. The sections were incubated with Vectastain blocking serum (horse) (ABC kit, Vector labs, Vector Labs, Peterborough, UK) then incubated overnight with primary antibody at room temperature: Rabbit polyclonal anti-ENPEP (Aminopeptidase A) (Cat no. HPA005128, Sigma Aldrich, Dorset, UK) was diluted 1 in 75 and mouse monoclonal CD13 (AP-N) (cat no. ab7417, Abcam, Cambridge, UK) was diluted 1 in 400. All sections were then incubated with biotinylated Universal Antibody followed by VectaElite ABC complex and with 3,3'-diaminobenzidine (DAB) for 7 minutes. After incubation with DAB, sections were washed in water and immersed in copper sulphate DAB enhancer for 4 minutes, counterstained with haematoxylin Gill II for 15 seconds, dehydrated, cleared and mounted in Clearium.

Statistical analysis

Parametric statistical tests were used for comparisons between groups and ANOVA with Bonferroni post-hoc test was used for multiple-group comparisons. Pearson analysis was used to assess the correlation between pairs of variables. Statistical tests were performed using SPSS version 23. P-values < 0.05 were considered statistically significant.

Results

Study cohort

The control (mean age-at-death ± SD = 78.5 ± 10 years and post-mortem (PM) delay ± SD = 43.8 ± 36 hours) and AD cohorts (mean age-at-death ± SD = 78.5 years ± 9.7 years and post-mortem delay ± SD = 45.0 hours ± 25 hours) were matched closely for age-at-death and PM delay (Table 1). Ang-II and Ang-III levels (Supplementary Figure 1), and AP-A and AP-N level and enzyme activity (Supplementary Figure 2) did not vary according to age-at-death or PM delay.

The control and AD cohorts varied with regard to the ratio of male: female with a higher ratio of males in the control (F 22: M 37) and females in the AD group (F 55: M 35) (Table 1). Ang-II and Ang-III level did not differ between genders although a non-significant trend of higher Ang-II was observed in females compared to males in both the control and AD group (Supplementary Figure 3). Ang-III level, and AP-A and AP-N enzyme activity and protein levels were not influenced by gender (Supplementary Figure 3).

Angiotensin II level is increased in Alzheimer's disease and does not correlate with parenchymal Aβ and tau load

Ang-II level was significantly elevated in AD (mean ± SEM = 58.30 ± 2.33 pg/mg total protein) compared with controls (mean ± SEM = 39.49 ± 3.34 pg/mg total protein) ($P < 0.0001$) (Fig. 1A). When the cases were stratified according to Braak tangle stage, an indicator of disease severity, Ang-II level was significantly increased in Braak stage V-VI (mean ± SEM = 56.42 ± 2.62 pg/mg total protein) compared to Braak stage 0-II (mean ± SEM = 38.63 ± 3.40 pg/mg total protein) ($P < 0.001$) (Fig. 1B). Ang-II level was increased in Braak stage III-IV (mean ± SEM = 49.96 ± 4.45 pg/mg total protein) but was not significantly different to Braak stage 0-II. Ang-II level did not correlate with insoluble Aβ level ($r = 0.12$; $P = 0.20$) (Fig. 1C) or parenchymal tau load ($r = 0.16$; $P = 0.06$) (Fig. 1D).

Angiotensin III level is increased in Alzheimer's disease and correlates strongly with parenchymal Aβ and tau load

Ang-III level was significantly elevated in AD (mean ± SEM = 154.79 ± 2.90 ng/mg total protein) compared with controls (mean ± SEM = 127.01 ± 3.27 ng/mg total protein) ($P < 0.0001$) (Fig. 2A). When the cases were stratified according to Braak tangle stage, an indicator of disease severity, the level of Ang-III was significantly increased in Braak stage V-VI (mean ± SEM = 157.49 ± 3.18 ng/mg total protein) compared to Braak stage 0-II (mean ± SEM = 127.66 ± 3.99 ng/mg total protein) ($P < 0.0001$) (Fig. 2B). Ang-III level was unchanged between Braak stage 0-II and III-IV (mean ± SEM = 134.19 ± 4.28 ng/mg total protein). A strongly significant positive correlation was observed between the level of Ang-III and the concentration of Aβ ($r = 0.49$, $P < 0.0001$) (Fig. 2C) and between Ang-III and parenchymal tau load (0.28, $P < 0.01$) (Fig. 2D). Multiple regression analysis (Ang-III as the dependent variable and Aβ and ptau load as the predictors) indicates that Aβ alone is a strong independent predictor of Ang-III ($P < 0.0001$) whereas ptau is not ($P = 0.072$).

Aminopeptidase-A level is reduced in AD

Aminopeptidase-A (AP-A) is the rate-limiting enzyme in the production of Ang-III. AP-A level was significantly reduced in AD (mean ± SEM = 1285.45 ± 47.35 ng/mg total protein) compared to controls (mean ± SEM = 1696 ± 66.35 ng/mg total protein) ($P < 0.0001$) (Fig. 3A) and was significantly lower in Braak stage V-VI (mean ± SEM = 1255.67 ± 47.96 ng/mg total protein) compared to Braak stage 0-II (mean ± SEM = 1732.5 ± 86.96 ng/mg total protein) ($P < 0.0001$). No difference was observed between Braak stage III-IV (mean ± SEM = 1632.52 ± 97.73 ng/mg total protein) and 0-II. Despite the reduction in AP-A level, a trend towards higher AP-A activity was observed in AD (mean ± SEM = 2.11 ± 0.23 mMol/hour/mg protein) compared to controls (mean ± SEM = 1.73 ± 0.18 mMol/hour/mg protein) (Fig. 3C) and in relation to Braak tangle stage (BS) (mean ± SEM = 1.75 ± 0.20 mMol/hour/mg protein in BS 0-II; 1.92 ± 0.43 mMol/hour/mg protein in BS III-IV and 2.08 ± 0.24 mMol/hour/mg protein in BS V-VI) (Fig. 3D) although neither reached statistical significance.

Aminopeptidase-N level is unchanged but enzyme activity is reduced in AD

AP-N level was unchanged in AD (mean ± SEM 55.85 ± 1.91 ng/mg total protein) compared to controls (mean ± SEM 53.16 ± 2.05 ng/mg total protein) (Fig. 4A) and did not vary according to Braak tangle stage (mean ± SEM 52.71 ± 2.40 ng/mg total protein in BS 0-II ; 53.2 ± 3.18 ng/mg total protein in BS III-IV and 56.27 ± 2.10 ng/mg total protein in BS V-VI) (Fig. 4B). AP-N activity was significantly lower in AD (mean ± SEM 2.24 ± 0.16 mMol/hour/mg total protein) compared with age-matched controls (mean ± SEM 2.86 ± 0.22 mMol/hour/mg protein) ($P < 0.05$) (Fig. 3C). A trend towards reduced AP-N activity in relation to increasing Braak stage severity was observed (mean ± SEM 2.89 ± 0.25 mMol/hour/mg protein in BS 0-II; 2.39 ± 0.35 mMol/hour/mg protein and 2.24 ± 0.17 mMol/hour/mg protein in BS V-VI), however, there were no significant differences between Braak tangle stage groups.

Aminopeptidase-A localisation in human brain tissue

AP-A immunolabeling of the vasculature was observed within the temporal neocortex and subiculum in both AD and controls (Figure 5). Perivascular AP-A labeling was also present in areas of focal white matter ischaemic damage (Figure 5). AP-A labeling of macrophages and microglia was observed, particularly in association with Aβ plaques and occasional neuronal labeling was present in both AD and controls. A similar staining pattern was observed in the frontal and parietal neocortex (not shown).

Aminopeptidase-N localisation in human brain tissue

AP-N neuronal labeling was observed within the frontal neocortex, CA2 region of the hippocampus, temporal neocortex and parietal neocortex in AD (Figure 6a-d). A similar distribution of AP-N was observed in controls, albeit with much lower intensity (Figure 6e-h). AP-N immunolabeling was not observed in the vasculature.

Discussion

In the present study we measured Ang-II and Ang-III levels in relation to pathological markers of Alzheimer's disease. Our data indicate that Ang-II and Ang-III levels are both increased in AD but that Ang-III levels correlate strongly with parenchymal Aβ and tau load whereas Ang-II levels did not. We also found dysregulation of the APA/Ang-III/APN/Ang-IV/AT4R pathway in AD. AP-A (responsible for the conversion of Ang-II to Ang-III) levels were significantly reduced in AD but enzyme activity was unaltered. AP-N (responsible for the conversion of Ang-III to Ang-IV) activity was reduced in AD despite AP-N protein levels remaining unchanged. Together, these data suggest dysregulation of the APA/Ang-III/APN/Ang-IV/AT4R pathway might contribute to elevated Ang-III in

AD due to increased conversion of Ang-II to Ang-III and reduced metabolism of Ang-III to Ang-IV. Together these data suggest that Ang-III level, and the enzymes involved in its processing, may contribute to the pathogenesis of AD.

Hyperactivity of the classical axis of brain RAS (ACE-1/Ang-II/AT1R) via Ang-II-mediated signalling through the AT1R receptor is implicated in the pathogenesis of AD (reviewed in [5]). We have previously reported that ACE-1 activity (rate-limiting in Ang-II production) is increased in human post-mortem brain tissue in association with higher parenchymal Aβ load [8]. In the present study, we measured Ang-II and Ang-III levels in human post-mortem brain tissue in AD. Ang-II was elevated in AD but did not correlate with Aβ and tau levels. In contrast, Ang-III was significantly increased in AD and correlated strongly with both Aβ level and parenchymal tau load. Ang-III has been shown to bind with equipotency to AT1r and AT2r and share many of the physiological functions of Ang-II and studies have shown that orally administered AP-A inhibitors, which presumably reduce the level of Ang-III, are cardio-protective [25-28]. It is conceivable that Ang-III, rather than Ang-II, may be the central effector in the classical axis of brain RAS.

We have also explored the pathways responsible for Ang-III metabolism, commonly referred to as the APA/Ang-III/APN/Ang-IV/AT4R pathway, which involves AP-A-mediated conversion of Ang-II to Ang-III and further downstream processing of Ang-III by AP-N to Ang-IV. Our data suggest that increased Ang-III is associated with a non-significant trend towards increased AP-A activity i.e. increased conversion of Ang-II to Ang-III, in combination with significantly reduced AP-N activity i.e. reduced conversion of Ang-III to Ang-IV.

It is currently unclear why there is a discrepancy between AP-A level (significantly reduced) and activity (non-significantly elevated) but this could indicate that there is post-translational modification in AD that enhances that activity of AP-A relative to the reduced AP-A protein level. These findings in brain tissue are in contrast to previous studies showing a gender-specific reduction (lower in males) in plasma and serum AP-A activity in AD [32, 33]; whether this is due to differences

between the brain and periphery (as we have also observed for ACE-1 activity in brain tissue [8, 9, 35]) remains unknown. Our immunohistochemical studies revealed that AP-A was mostly localised within microglia surrounding Aβ plaques in AD suggesting that there may be upregulation of a specific pool of AP-A in conjunction with an immune response associated with AD pathology. Alternatively, the trend towards increased AP-A activity in AD may simply reflect a compensatory response of AP-A in the presence of increasing Ang-II level. AP-A generates highly amyloidogenic and neurotoxic N-terminal truncated and pyroglutamated (AβpE3) Aβ42 species that could also contribute directly to AD pathogenesis [40, 41].

AP-N activity was significantly reduced in AD in contrast to protein levels that either remained unchanged (determined by ELISA) or appeared to be elevated (immunohistochemical analysis). A reduction in AP-N activity could however potentially mediate reduced conversion of Ang-III to Ang-IV resulting in elevated Ang-III level, which would not only impact on disease pathology but could also influence downstream signalling pathways involved in cognition - Ang-IV mediated inhibition of its receptor IRAP is heavily implicated in learning and memory processes [30, 31]. Serum and plasma AP-N activities are also reduced in AD [32, 33]. Together, our data indicate that Ang-III may be increased in AD as a result of dysregulation of the APA/Ang-III/APN/Ang-IV/AT4R pathway and could contribute not only to disease pathology but also to memory impairment.

Emerging data has identified a number of regulatory pathways within brain RAS that are involved in the metabolism of Ang-II and counter-regulate the activity of the 'classical' RAS axis (reviewed in [4]). ACE-2 converts Ang-II to Ang 1-7, which via the MAS receptor counter-regulates the actions of the classical axis, known as the ACE2/Ang(1-7)/MASR pathway (reviewed in [42, 43]). We recently showed that ACE-2 activity is highly significantly reduced in AD and is inversely correlated with both Aβ level and tau parenchymal load [44]. Ang 1-7 (via the MAS receptor) is also implicated in long-term potentiation [45] and therefore loss of the activity within the ACE2/Ang(1-

7)/MASR pathway may not only be associated with disease pathology, but as for Ang-IV, may adversely impact on learning and memory processes.

These data indicate that elevated Ang-III level in AD is strongly linked with disease pathology and is possibly associated with dysregulation of the APA/APN/Ang-IV/AT4R pathway within brain RAS. Longitudinal studies, such as those described by Gard et al. [32] who measured changes in serum aminopeptidases activity in healthy and mild-moderate AD at various stages of disease, will be useful in determining whether changes within these RAS pathways contribute to the early pathological changes in AD.

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Disclosure/Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

JSM and PK were responsible for the conception and design of experiments; JSM, EH and LP were responsible for acquisition of data; JSM analyzed and interpreted the data; JSM drafted the

manuscript; PK and JSM revised and reviewed the final article for intellectual content and final approval.

References

[1] Phillips MI, de Oliveira EM (2008) Brain renin angiotensin in disease. *J Mol Med (Berl)* **86**, 715-722.

[2] Wright JW, Harding JW (2011) Brain renin-angiotensin--a new look at an old system. *Prog Neurobiol* **95**, 49-67.

[3] Wright JW, Harding JW (2013) The brain renin-angiotensin system: a diversity of functions and implications for CNS diseases. *Pflugers Arch* **465**, 133-151.

[4] Wright JW, Kawas LH, Harding JW (2013) A Role for the Brain RAS in Alzheimer's and Parkinson's Diseases. *Front Endocrinol (Lausanne)* **4**, 158.

[5] Kehoe PG, Miners S, Love S (2009) Angiotensins in Alzheimer's disease - friend or foe? *Trends Neurosci* **32**, 619-628.

[6] Tian M, Zhu D, Xie W, Shi J (2012) Central angiotensin II-induced Alzheimer-like tau phosphorylation in normal rat brains. *FEBS Lett* **586**, 3737-3745.

[7] Zhu D, Shi J, Zhang Y, Wang B, Liu W, Chen Z, Tong Q (2011) Central angiotensin II stimulation promotes beta amyloid production in Sprague Dawley rats. *PLoS One* **6**, e16037.

[8] Miners JS, Ashby E, Van Helmond Z, Chalmers KA, Palmer LE, Love S, Kehoe PG (2008) Angiotensin-converting enzyme (ACE) levels and activity in Alzheimer's disease, and relationship of perivascular ACE-1 to cerebral amyloid angiopathy. *Neuropathol Appl Neurobiol* **34**, 181-193.

[9] Miners S, Ashby E, Baig S, Harrison R, Tayler H, Speedy E, Prince JA, Love S, Kehoe PG (2009) Angiotensin-converting enzyme levels and activity in Alzheimer's disease: differences in brain and CSF ACE and association with ACE1 genotypes. *Am J Transl Res* **1**, 163-177.

[10] Ashby EL, Kehoe PG (2013) Current status of renin-aldosterone angiotensin system-targeting anti-hypertensive drugs as therapeutic options for Alzheimer's disease. *Expert Opin Investig Drugs* **22**, 1229-1242.

[11] Corbett A, Pickett J, Burns A, Corcoran J, Dunnett SB, Edison P, Hagan JJ, Holmes C, Jones E, Katona C, Kearns I, Kehoe P, Mudher A, Passmore A, Shepherd N, Walsh F, Ballard C (2012) Drug repositioning for Alzheimer's disease. *Nat Rev Drug Discov* **11**, 833-846.

[12] Chauvel EN, Llorens-Cortes C, Coric P, Wilk S, Roques BP, Fournie-Zaluski MC (1994) Differential inhibition of aminopeptidase A and aminopeptidase N by new beta-amino thiols. *J Med Chem* **37**, 2950-2957.

[13] Healy DP, Wilk S (1993) Localization of immunoreactive glutamyl aminopeptidase in rat brain. II. Distribution and correlation with angiotensin II. *Brain Res* **606**, 295-303.

[14] Ramirez M, Arechaga G, Garcia S, Sanchez B, Lardelli P, de Gandarias JM (1990) Mn2(+)-activated aspartate aminopeptidase activity, subcellular localization in young and adult rat brain. *Brain Res* **522**, 165-167.

[15] Rich DH, Moon BJ, Harbeson S (1984) Inhibition of aminopeptidases by amastatin and bestatin derivatives. Effect of inhibitor structure on slow-binding processes. *J Med Chem* **27**, 417-422.

[16] Mukoyama M, Nakajima M, Horiuchi M, Sasamura H, Pratt RE, Dzau VJ (1993) Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven-transmembrane receptors. *J Biol Chem* **268**, 24539-24542.

[17] Murphy TJ, Alexander RW, Griendling KK, Runge MS, Bernstein KE (1991) Isolation of a cDNA encoding the vascular type-1 angiotensin II receptor. *Nature* **351**, 233-236.

[18] Abhold RH, Sullivan MJ, Wright JW, Harding JW (1987) Binding, degradation and pressor activity of angiotensins II and III after aminopeptidase inhibition with amastatin and bestatin. *J Pharmacol Exp Ther* **242**, 957-962.

[19] Wright JW, Jensen LL, Roberts KA, Sardinia MF, Harding JW (1989) Structure-function analyses of brain angiotensin control of pressor action in rats. *Am J Physiol* **257**, R1551-1557.

[20] Wright JW, Morseth SL, Abhold RH, Harding JW (1985) Pressor action and dipsogenicity induced by angiotensin II and III in rats. *Am J Physiol* **249**, R514-521.

[21] Wright JW, Sullivan MJ, Petersen EP, Harding JW (1985) Brain angiotensin II and III binding and dipsogenicity in the rabbit. *Brain Res* **358**, 376-379.

[22] Phillips MI (1987) Functions of angiotensin in the central nervous system. *Annu Rev Physiol* **49**, 413-435.

[23] Saavedra JM (1992) Brain and pituitary angiotensin. *Endocr Rev* **13**, 329-380.

[24] Zini S, Fournie-Zaluski MC, Chauvel E, Roques BP, Corvol P, Llorens-Cortes C (1996) Identification of metabolic pathways of brain angiotensin II and III using specific aminopeptidase inhibitors: predominant role of angiotensin III in the control of vasopressin release. *Proc Natl Acad Sci U S A* **93**, 11968-11973.

[25] Gao J, Marc Y, Iturrioz X, Leroux V, Balavoine F, Llorens-Cortes C (2014) A new strategy for treating hypertension by blocking the activity of the brain renin-angiotensin system with aminopeptidase A inhibitors. *Clin Sci (Lond)* **127**, 135-148.

[26] Llorens-Cortes C (2014) [Orally active aminopeptidase A inhibitors reduce blood pressure: a new strategy for treating hypertension]. *Biol Aujourdhui* **208**, 217-224.

[27] Marc Y, Gao J, Balavoine F, Michaud A, Roques BP, Llorens-Cortes C (2012) Central antihypertensive effects of orally active aminopeptidase A inhibitors in spontaneously hypertensive rats. *Hypertension* **60**, 411-418.

[28] Marc Y, Llorens-Cortes C (2011) The role of the brain renin-angiotensin system in hypertension: implications for new treatment. *Prog Neurobiol* **95**, 89-103.

[29] Palmieri FE, Bausback HH, Ward PE (1989) Metabolism of vasoactive peptides by vascular endothelium and smooth muscle aminopeptidase M. *Biochem Pharmacol* **38**, 173-180.

[30] Albiston AL, Diwakarla S, Fernando RN, Mountford SJ, Yeatman HR, Morgan B, Pham V, Holien JK, Parker MW, Thompson PE, Chai SY (2011) Identification and development of specific inhibitors for insulin-regulated aminopeptidase as a new class of cognitive enhancers. *Br J Pharmacol* **164**, 37-47.

[31] Albiston AL, Morton CJ, Ng HL, Pham V, Yeatman HR, Ye S, Fernando RN, De Bundel D, Ascher DB, Mendelsohn FA, Parker MW, Chai SY (2008) Identification and characterization of a new cognitive enhancer based on inhibition of insulin-regulated aminopeptidase. *FASEB J* **22**, 4209-4217.

[32] Gard PR, Fidalgo S, Lotter I, Richardson C, Farina N, Rusted J, Tabet N (2017) Changes of renin-angiotensin system-related aminopeptidases in early stage Alzheimer's disease. *Exp Gerontol* **89**, 1-7.

[33] Puertas Mdel C, Martinez-Martos JM, Cobo M, Lorite P, Sandalio RM, Palomeque T, Torres MI, Carrera-Gonzalez MP, Mayas MD, Ramirez-Exposito MJ (2013) Plasma renin-angiotensin system-regulating aminopeptidase activities are modified in early stage Alzheimer's disease and show gender differences but are not related to apolipoprotein E genotype. *Exp Gerontol* **48**, 557-564.

[34] Montine TJ, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Dickson DW, Duyckaerts C, Frosch MP, Masliah E, Mirra SS, Nelson PT, Schneider JA, Thal DR, Trojanowski JQ, Vinters HV, Hyman BT, National Institute on A, Alzheimer's A (2012) National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. *Acta Neuropathol* **123**, 1-11.

[35] Miners JS, van Helmond Z, Raiker M, Love S, Kehoe PG (2010) ACE variants and association with brain Abeta levels in Alzheimer's disease. *Am J Transl Res* **3**, 73-80.

[36] van Helmond Z, Miners JS, Kehoe PG, Love S (2010) Higher soluble amyloid beta concentration in frontal cortex of young adults than in normal elderly or Alzheimer's disease. *Brain Pathol* **20**, 787-793.

[37] Ballard CG, Chalmers KA, Todd C, McKeith IG, O'Brien JT, Wilcock G, Love S, Perry EK (2007) Cholinesterase inhibitors reduce cortical Abeta in dementia with Lewy bodies. *Neurology* **68**, 1726-1729.

[38] Chalmers KA, Wilcock GK, Vinters HV, Perry EK, Perry R, Ballard CG, Love S (2009) Cholinesterase inhibitors may increase phosphorylated tau in Alzheimer's disease. *J Neurol* **256**, 717-720.

[39] Aoyagi T, Tobe H, Kojima F, Hamada M, Takeuchi T, Umezawa H (1978) Amastatin, an inhibitor of aminopeptidase A, produced by actinomycetes. *J Antibiot (Tokyo)* **31**, 636-638.

[40] Antonios G, Saiepour N, Bouter Y, Richard BC, Paetau A, Verkkoniemi-Ahola A, Lannfelt L, Ingelsson M, Kovacs GG, Pillot T, Wirths O, Bayer TA (2013) N-truncated Abeta starting with position four: early intraneuronal accumulation and rescue of toxicity using NT4X-167, a novel monoclonal antibody. *Acta Neuropathol Commun* **1**, 56.

[41] Brannstrom K, Ohman A, Nilsson L, Pihl M, Sandblad L, Olofsson A (2014) The N-terminal region of amyloid beta controls the aggregation rate and fibril stability at low pH through a gain of function mechanism. *J Am Chem Soc* **136**, 10956-10964.

[42] Jiang T, Gao L, Lu J, Zhang YD (2013) ACE2-Ang-(1-7)-Mas Axis in Brain: A Potential Target for Prevention and Treatment of Ischemic Stroke. *Curr Neuropharmacol* **11**, 209-217.

[43] Xu P, Sriramula S, Lazartigues E (2011) ACE2/ANG-(1-7)/Mas pathway in the brain: the axis of good. *Am J Physiol Regul Integr Comp Physiol* **300**, R804-817.

[44] Kehoe PG, Wong S, Al Mulhim N, Palmer LE, Miners JS (2016) Angiotensin-converting enzyme 2 is reduced in Alzheimer's disease in association with increasing amyloid-beta and tau pathology. *Alzheimers Res Ther* **8**, 50.

[45] Hellner K, Walther T, Schubert M, Albrecht D (2005) Angiotensin-(1-7) enhances LTP in the hippocampus through the G-protein-coupled receptor Mas. *Mol Cell Neurosci* **29**, 427-435.

Figure Legends

Figure 1. Angiotensin-II (Ang-II) level is increased in the mid-frontal cortex in Alzheimer's disease (AD). (**A**) Bar chart showing significantly elevated Ang-II levels in AD compared to age-matched controls ($P < 0.0001$ using an unpaired *t*-test). (**B**) Bar chart showing increased Ang-II level in relation to disease severity, indicated by Braak tangle stage. Angiotensin-II level was significantly increased in Braak stage V-VI compared to 0-II ($P < 0.001$ using a one-way ANOVA with Bonferroni post-hoc analysis). In A and B the bars represent the mean and SEM. (**C-D**) Scatterplots showing a trend towards positive correlations between Ang-II level and (C) guanidine-HCl-extracted insoluble amyloid-beta (Aβ) level and (D) parenchymal tau load. The best-fit linear regression lines (inner line) and 95% confidence intervals are superimposed. Each dot represents a single case. The green circles indicate controls and the red circles indicate AD cases.

Figure 2. Angiotensin-III (Ang-III) level is increased in the mid-frontal cortex in Alzheimer's disease (AD). (**A**) Bar chart showing significantly elevated Ang-III levels in AD compared to age-matched controls ($P < 0.0001$ using unpaired *t*-test). (**B**) Bar chart showing increased angiotensin-III level in relation to disease severity, indicated by Braak tangle stage. Ang-III level was significantly increased in Braak stage V-VI compare to 0-II ($P < 0.0001$ using one-way ANOVA with Bonferroni post-hoc analysis). In A and B the bars represent the mean and SEM. (**C-D**) Scatterplots showing highly significant positive correlations between Ang-III level and (C) insoluble Aβ level and (D) parenchymal tau load. The best-fit linear regression lines (inner line) and 95% confidence intervals are superimposed. Each dot represents a single case. The green circles indicate controls and the red circles indicate AD cases.

Figure 3. Aminopeptidase-A (AP-A) protein levels are significantly reduced in Alzheimer's disease (AD) but enzyme activity is unaltered. (**A**) Bar charts showing significantly reduced AP-A level in AD ($P < 0.0001$ using an unpaired *t*-test) (**B**) AP-A level was significantly reduced in Braak stage V-VI compared to Braak stage 0-II ($P < 0.0001$ using one-way ANOVA with Bonferroni post-hoc analysis). (**C-D**) Bar charts show a trend towards higher AP-A enzyme activity in AD in relation to disease

severity (despite reduced protein level), indicated by highest activity in Braak tangle stage V-VI, although the difference was not statistically significant. Bars represent the mean and SEM.

Figure 4. Aminopeptidase-N (AP-N) enzyme activity is reduced in Alzheimer's disease (AD) but protein levels are unaltered. (**A-B**) Bar charts showing unchanged AP-N levels in AD compared to age-matched controls in relation to disease stage severity. (**C-D**) Bar charts showing significantly lower AP-N activity in AD compared to age-matched controls ($P < 0.05$ using an unpaired *t*-test) and in relation to disease severity, indicated by lower activity in Braak tangle stage V-VI, although this difference was not statistically significant. Bars represent the mean and SEM.

Figure 5. Aminopeptidase-A (AP-A) localisation in human brain tissue. AP-A distribution in the temporal neocortex (a and b, scale bars = 50μm) and subiculum (c, scale bar = 100μm) of an Alzheimer's disease (AD) patient. AP-A also labelled cells in the temporal neocortex (d and f, scale bars = 50μm) and an area of focal white matter ischaemic damage (e, scale bar = 100μm) in controls. Prominent vascular labeling (g, scale bar = 50μm) and labeling was also present in the subiculum (h, scale bar = 100μm) of controls. AP-A immunolabeling was observed in macrophages and microglia in both AD cases and controls. Staining was also seen in these cell types in association with Aβ plaques. Occasional neuronal immunolabeling was seen in both AD cases and controls. High levels of labeling were seen in an area of focal white matter ischaemic damage (d and f) while prominent vascular labeling was present in some subjects.

Figure 6. Aminopeptidase-N (AP-N) localisation in human brain tissue. AP-N distribution in the frontal neocortex (a), CA2 region of the hippocampus (b), temporal neocortex (c) and parietal neocortex (d) of an Alzheimer's disease (AD) patient. AP-N also labelled some neurons, albeit with less intensity, in the frontal neocortex (e), CA2 region of the hippocampus (f), temporal neocortex (g) and parietal neocortex (h) of control subjects. No vessel staining was observed in any of the cases examined. Scale bar = 50μm for all sections.

Table 1. Summary of demographics of control and AD cases

	Age-at-death (years ± SD)	Gender (Females: Males)	Post-mortem delay (hours ± SD)	Braak tangle stage		
				0-II	III-IV	V-VI
Controls (n = 59)	78.5 ± 10.0	22:37	43.8 ± 36	41	15	0
AD (n = 90)	78.5 ± 9.7	55:35	45.0 ± 25	0	13	77

Braak Tangle stage score unavailable for 3 control case

Supplementary Table 1: MRC identifiers

Diagnosis	MRC identifier	Age-at-death (h)	Gender	Post-Mortem delay (h)	Braak Tangle stage
Control	BBN_8639	62	M	4	0
Control	BBN_8644	84	M	72	3
Control	BBN_8651	95	F	46	2
Control	BBN_8671	78	F	24	2
Control	BBN_8682	83	M	80	3
Control	BBN_8684	71	M	25	1
Control	BBN_8691	82	F	35	3
Control	BBN_8700	64	M	12	2
Control	BBN_8702	58	M	20	0
Control	BBN_8703	64	M	16	0
Control	BBN_8706	72	M	42	1
Control	BBN_8707	80	M	106	3
Control	BBN_8708	90	M	45	2
Control	BBN_8709	83	M	86	2
Control	BBN_8712	81	F	103	2
Control	BBN_8714	64	M	23	2
Control	BBN_8717	77	M	55	1
Control	BBN_8722	78	M	12	2
Control	BBN_8723	80	M	67	3
Control	BBN_8725	73	M	36	2
Control	BBN_8728	88	F	62	2
Control	BBN_8731	70	M	50	2
Control	BBN_8732	76	F	106	2
Control	BBN_8735	88	F	72	0
Control	BBN_8739	93	F	18	2
Control	BBN_8741	80	F	92	0

Control	BBN_8749	88	F	28	2
Control	BBN_8751	82	M	30	2
Control	BBN_8756	84	M	48	3
Control	BBN_8757	90	M	48	2
Control	BBN_8759	75	M	48	2
Control	BBN_8768	78	F	22	1
Control	BBN_8770	89	F	15	2
Control	BBN_8776	73	M	33	1
Control	BBN_8779	69	M	66	2
Control	BBN_8835	73	F	59	1
Control	BBN_8883	90	M	40	3
Control	BBN_8888	89	M	91	2
Control	BBN_8898	83	F	24	2
Control	BBN_8923	82	M	3	2
Control	BBN_8949	79	M	24	.
Control	BBN_8956	43	F	12	.
Control	BBN_8957	76	F	12	.
Control	BBN_8961	84	F	17	1
Control	BBN_8964	82	F	37	2
Control	BBN_8966	53	M	7	3
Control	BBN_8980	72	F	24	0
Control	BBN_8983	78	M	48	1
Control	BBN_9012	81	M	3	2
Control	BBN_9016	82	M	56	2
Control	BBN_9028	76	M	23	2
Control	BBN_9038	82	F	96	3
Control	BBN_9086	77	M	10	3
Control	BBN_9092	75	M	6	3
Control	BBN_9217	93	F	53	3

Control	BBN_9256	84	F	.	2
Control	BBN_9292	73	M	35	3
Control	BBN_9299	90	M	6	2
Control	BBN_9311	93	M	38	3
AD	BBN_8819	89	F	71	5
AD	BBN_8825	78	F	77	6
AD	BBN_8834	78	F	9	5
AD	BBN_8839	82	M	69	5
AD	BBN_8841	81	F	80	3
AD	BBN_8842	81	F	42	6
AD	BBN_8848	77	F	43	4
AD	BBN_8852	71	F	67	5
AD	BBN_8853	96	F	53	4
AD	BBN_8857	87	F	72	5
AD	BBN_8870	87	F	67	5
AD	BBN_8870	79	F	70	3
AD	BBN_8871	88	F	79	6
AD	BBN_8885	68	M	28	5
AD	BBN_8886	81	M	29	4
AD	BBN_8892	91	F	70	5
AD	BBN_8905	72	M	61	4
AD	BBN_8906	78	F	35	6
AD	BBN_8912	82	F	24	6
AD	BBN_8915	85	M	58	4
AD	BBN_8917	91	M	43	3
AD	BBN_8918	89	F	82	5
AD	BBN_8930	70	F	25	6
AD	BBN_8947	78	F	4	5
AD	BBN_8954	69	M	48	5

AD	BBN_8958	74	M	50	5
AD	BBN_8969	95	M	48	3
AD	BBN_8997	74	F	12	6
AD	BBN_9005	89	F	4	6
AD	BBN_9026	79	M	28	6
AD	BBN_9031	85	M	66	6
AD	BBN_9050	90	F	21	4
AD	BBN_9052	57	F	24	5
AD	BBN_9061	54	F	24	6
AD	BBN_9076	84	F	20	5
AD	BBN_9095	79	F	72	5
AD	BBN_9102	78	M	21	3
AD	BBN_9106	93	M	20	6
AD	BBN_9112	74	F	53	5
AD	BBN_9113	81	F	59	5
AD	BBN_9114	82	M	64	4
AD	BBN_9119	80	M	49	5
AD	BBN_9122	83	F	5	5
AD	BBN_9123	74	F	35	5
AD	BBN_9125	84	F	23	6
AD	BBN_9132	80	M	5	5
AD	BBN_9136	77	F	26	6
AD	BBN_9156	79	M	39	5
AD	BBN_9162	63	M	43	6
AD	BBN_9165	60	F	68	6
AD	BBN_9167	79	F	48	5
AD	BBN_9179	64	M	9	6
AD	BBN_9182	74	M	24	5
AD	BBN_9123	85	F	49	5

AD	BBN_9188	68	F	87	6
AD	BBN_9189	78	F	21	6
AD	BBN_9193	79	M	84	5
AD	BBN_9194	89	F	39	5
AD	BBN_9198	77	F	14	6
AD	BBN_9200	84	M	64	5
AD	BBN_9201	65	M	90	6
AD	BBN_9205	85	F	85	6
AD	BBN_9207	80	F	71	6
AD	BBN_9209	73	F	38	5
AD	BBN_9212	65	F	22	6
AD	BBN_9221	68	M	61	6
AD	BBN_9222	90	M	32	6
AD	BBN_9243	88	F	75	5
AD	BBN_9248	83	F	85	6
AD	BBN_9259	47	F	54	6
AD	BBN_9261	83	M	48	5
AD	BBN_9263	74	M	48	5
AD	BBN_9265	60	F	5	6
AD	BBN_9269	83	M	99	4
AD	BBN_9274	78	M	49	6
AD	BBN_9275	87	M	36	6
AD	BBN_9284	76	F	44	6
AD	BBN_9291	62	M	25	6
AD	BBN_9293	78	M	50	6
AD	BBN_9295	85	M	50	6
AD	BBN_9256	98	F	21	5
AD	BBN_9298	83	F	32	6
AD	BBN_9301	84	F	11	5

AD	BBN_9303	69	M	12	5
AD	BBN_9304	61	M	38	5
AD	BBN_9309	88	F	88	5
AD	BBN_9310	85	F	102	6
AD	BBN_9315	67	F	24	6
AD	BBN_9320	87	F	28	6
AD	BBN_9323	84	F	21	6

Supplementary Figure 1. Angiotensin-II (Ang-II) and angiotensin-III (Ang-III) levels are not influenced by age-at-death or post-mortem (PM) delay. (**A-B**) Scatterplots showing no significant correlation between Ang-II and age-at-death and PM delay. (**C-D**) Scatterplots showing no significant correlation between Ang-III and age-at-death and PM delay. The best-fit linear regression lines (inner line) and 95% confidence intervals are superimposed. Each dot represents a single case. The green circles indicate controls and the red circles indicate AD cases.

Supplementary Figure 2. Aminopeptidase-A (AP-A) and aminopeptidase-N (AP-N) level and enzyme activity are not influenced by age-at-death or post-mortem (PM) delay. (**A-D**) Scatterplots showing no significant correlation between AP-A level or AP-A enzyme activity with age-at-death and PM delay. (**E-H**) Scatterplots showing no significant correlation between AP-A level or AP-A enzyme activity with age-at-death and PM delay. The best-fit linear regression lines (inner line) and 95% confidence intervals are superimposed. Each dot represents a single case. The green circles indicate controls and the red circles indicate AD cases.

Supplementary Figure 3. Ang-II, Ang-III and AP-A and AP-N level and activity is not influenced by gender. Bar charts showing gender specific levels of (A) Ang-II and (B) Ang-III in AD and controls. Ang-II levels were higher in females in both AD and controls but the difference was not statistically significant; Ang-III levels are unaltered between genders. Bar charts showing gender specific (C-D) AP-A levels and activities and (E-F) AP-N levels and activities which were unaltered in AD and controls. The bars represent the mean and SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ and **** $P < 0.0001$